

OBSERVATIONS ON D'HERELLE'S BACTERIOPHAGE

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TWO PLATES

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INTRODUCTION

The bacteriophage of d'Herelle is, at the present time, defined by a limited number of properties. We are unable to make, without equivocation, even the first fundamental step in its classification, namely, assignment either to the chemical sciences or to the biologic sciences. We may say only that if the bacteriophage falls within the realms of chemistry, it should be classified with the enzymes; if it falls within the realms of biology, it should be classified with the filtrable viruses. Neither the enzyme, the filtrable virus, nor the bacteriophage is defined by any vast number of specific properties.

D'Herelle has, from the time of his original work, claimed that the bacteriophage was a living, ultramicroscopic organism, *Bacteriophagum intestinale*. His most recent and perhaps strongest argument for the truth of his contention might be summarized as follows: If any one characteristic of living things could be positively stated to be a property of all living things and the property of no nonliving substance, it would be necessary to show only that the bacteriophage possesses, or does not possess, this property. After a study of this question, he concludes that there is such a characteristic—namely, the ability of living matter to incorporate the inanimate material of a medium in which it is placed into its own living substance—or, to put the matter in his own words, the “power of assimilation in a heterogeneous medium.” There is also a second criterion, the power of adaptation. Although the evidence is not wholly incontrovertible, it seems more than probable that the bacteriophage possesses these properties.

Perhaps the greatest obstacle in the way of fixing the properties of these substances, and one which, if overcome, might aid us in fitting them into their proper niches in science, is the difficulty in securing unequivocal quantitative data. Qualitative data are a necessary prelude, but quantitative data must follow closely.

It is the aim of the present work, started in 1922, to study some of the properties of the bacteriophage as closely as possible from the quantitative point of view. It is necessary, as one phase in the study of the action of the bacteriophage, to observe its influence on living cells. Thus, to the bacteriophage, which we must regard as a variable, and which may or may not be a living thing, we are forced to add another variable, which is living—the bacterial cell. Although the study of such variables may not be characterized by the accuracy justly demanded by the chemist, it is the study of the influence of the living cell on the bacteriophage and the influence of the bacteriophage on the living cell which involves the crucial point of the whole problem, and this has formed the primary object of our study. Corollary to this, certain investigations have been carried out on the absorption of bacteriophage by bacterial cells, on the diffusion of the bacteriophage through semipermeable membranes, on the resistance of the bacterial cell to the lytic action of the bacteriophage, and on the specific antigenic properties of the lysed bacterial cell.

The original work of d'Herelle has been set forth at length in his book,¹ and has been extended somewhat in the English translation,² and further by his more recent work.³ D'Herelle's first observations were made in 1912 in connection with his studies of a locust disease. The work probably received an added stimulus through the work of Twort⁴ in 1915. D'Herelle has worked steadily on the bacteriophage since 1917, and his work is so well known that no space will be devoted to it. Bibliographic summaries of the work done have been given by Davison,⁵ Pico,⁶ Rhodes,⁷ and Kuttner.⁸ Since 1922, papers dealing with the bacteriophage have been numerous, and have added a great deal to our knowledge of the details of its mode of action and the influence of environmental conditions. A number of papers have been written on the nature of bacteriophage, some claiming proof of the enzymatic nature of the active agent, others supporting the theory of the living nature of the agent. The bacteriophage has been found to be so ubiquitous that the fact that it was only recently discovered is a credit to the astute mind of d'Herelle. Different types of bacteriophage exhibiting lytic properties for a large variety of bacteria have been reported, among the most recent being a bacteriophage active against the Klebs-Loeffler bacillus, reported

¹ *Le Bacteriophage, son Rôle dans l'Immunité*, 1921.

² *The Bacteriophage*, 1922.

³ *Immunity in Natural Infectious Disease*, 1924.

⁴ *Lancet*, 1915, 189, p. 1241.

⁵ *Abst. Bacteriol.*, 1922, 4, p. 159.

⁶ *Semana méd.*, 1922, 19, p. 415.

⁷ *Jour. Lab. & Clin. Med.*, 1922, 7, p. 288.

⁸ *Jour. Bacteriol.*, 1923, 8, p. 49.

by Blair.⁹ Evidence has been given to show that there are many types or strains of bacteriophage differing in their action against different species of bacteria, as well as against different strains of the same species. This has been made especially clear by Asheshov¹⁰ in a recent paper. These differences in the mode of action of different types of bacteriophage are of much significance, and it is only by the accumulation of data relating to these differences that it may be ascertained how far the data on one bacteriophage are fundamentally applicable to all.

EXPERIMENTAL

Quantitative Measurement.—Quantitative measurement of the potency, "virulence," or lytic action of a liquid endowed with the properties of the bacteriophage might conceivably be made by physical, chemical, or bacteriologic means. It is possible that chemical means will in time be devised for such measurement, and that physical measurements will follow. At present, however, because our knowledge of the bacteriophage is built around the lytic activity toward the bacterial cell, bacteriologic means of measurement are alone open to experimental use.

A suspension of living bacterial cells in which the cells are present in considerable concentration will, when inoculated on the surface of agar culture medium and incubated, form on the inoculated surface a solid mass of organisms piled perhaps a millimeter high. If, however, broth culturally sterile but containing bacteriophage in proper concentration be added to the suspension with which the agar is to be inoculated, and from this the growth of organisms be secured on an agar surface, there will result (provided the bacteriophage employed is active against the strain of bacteria used) a solid mass of organisms with here and there cleared zones or areas where there appears to have been no growth. To all outward appearances, there is a circular area on the agar to which no organisms had been inoculated. We know, however, from repeated experimentation, that the areas, as they will hereafter be designated, are due, not to lack of adequate inoculation, but to the lytic action exhibited at these foci by the bacteriophage. The number of such areas produced on a given field, taking into consideration the amount of bacteriophage-containing liquid used and the area of the field over which this number occurs, offers an index to the bacteriophage concentration in the original material.

D'Herelle, Maitland¹¹ and others have used this principle in making quantitative measurements of bacteriophage concentrations by streaking such mixtures of bacteriophage and bacteria to agar slants in more or less known concentrations. The resulting areas may be counted, and the bacteriophage concentration calculated accordingly.

There is another method, devised by d'Herelle, which has been variously used, involving the measurement of bacteriophage concentration by the use of the circumstance that even a single bacteriophage unit, if present in a tube of broth culture medium, will inhibit the growth or cause the eventual lysis of bacteria in that broth, provided that the bacteriophage is active against the bacterial strain used. Thus, a series of dilutions of the liquid containing bacteriophage may be made in a broth medium, the series inoculated with bacteria, and the highest dilution of the original bacteriophage-containing liquid inhibiting or causing the final destruction of the bacterial development ascertained.

⁹ Jour. Infect. Dis., 1924, 35, p. 401.

¹⁰ Jour. Infect. Dis., 1924, 34, p. 356.

¹¹ Brit. Jour. Exper. Pathol., 1922, 3, p. 173.

Agar plates instead of slants for the demonstration and enumeration of areas have been used to some extent, and this method is most recently and beautifully developed by Asheshov.¹⁰

Much of the quantitative work which has been done has been directed toward the securing of relative and comparative data for qualitative information. It is thus necessary, as this study is directed at more intimate quantitative data concerning one type of bacteriophage, that we take cognizance of certain errors in the foregoing methods as pertinent to the present work.

The use of drops, or of the amount of liquid taken up by a platinum loop, has been a procedure frequently followed in the elicitation of comparative quantitative information. In spite of the painstaking work of Miller,¹² it was our own experience that the same loop (2.2 mm.) might take up from 0.01 c.c. to 0.002 c.c., and that the amount taken up is dependent on viscosity and surface tension, which in turn are variable with physical factors ordinarily uncontrolled, notably the temperature. Furthermore, although the amount taken up by the loop when removed from a liquid into which it has been introduced might be neglected on account of the dilution secured, the amount removed when an agar surface is inoculated is uncertain. Volumes have been written concerning the drop as a unit of measurement.¹³ The size of a drop is primarily a function of the size of the opening, of the surface tension, and of the density of the liquid used. To be accurately duplicated, drops must fall at a slow rate from a definite opening "wet" only at the surface at which the drop is formed, the temperature must be constant, and the liquids must have similar densities and surface tensions. Any duplication secured without taking these precautions must be regarded as accidental. Using the ordinary precautions in a preliminary experiment in which drops were expected to give relatively correct results, the variation was from 20 to 37 drops per c.c.

Little need be said concerning the fallacy inherent in the use of turbidity as a criterion of bacterial growth from a quantitative standpoint. Experiments revealed that a bacterial concentration of 8×10^6 per c.c. was necessary before slight turbidity could be noticed; and that a very heavy turbidity represented from 175×10^6 to 400×10^6 organisms per c.c., beyond which only a marked difference could be detected with any degree of accuracy. This represents obviously only a small portion of the growth curve of bacteria. The nephelometer is not convenient when protection to the worker against infection is necessary, and does not allow of any measurement of the first few hours of bacterial growth, which are the most important.

An error in the dilution method for the estimation of bacteriophage concentration became apparent after making a considerable number of such estimations. By using tubes of broth containing just 9 c.c., a consecutive series of dilutions of a bacteriophage-containing liquid may be made, 1:10 or 10^{-1} , 1:100 or 10^{-2} , 10^{-3} , 10^{-3} , etc., the series inoculated with sensitive bacteria, incubated, and the end-point determined by a clear-cut reading of the growth; the inhibited and lysed tubes are perfectly clear, and subsequent dilutions show normal growth. This means a convenient and clear-cut procedure for a first approximation. However, in itself it is insufficient. A tube containing 8 bacteriophage units will exhibit the same complete inhibition as will a tube containing one bacteriophage unit. In a series: 10^{-1} 10^{-2} 10^{-3} 10^{-4} 10^{-5} 10^{-6} 10^{-7} 10^{-8} 10^{-9} dilutions of bacteriophage in which the 10^{-8} tube and all lower dilutions are clear after inoculation and incubation, and the 10^{-9} tube shows normal growth, one would say off-hand that there are between 100,000,000 and 1,000,000,000 bacteriophage units per c.c. in the original liquid. This is not legitimate, for

¹² Jour. Lab. & Clin. Med., 1924, 9, p. 404.

¹³ Harkins: Jour. Am. Chem. Soc., 1916, 38, Pt. 1, p. 228; Willows and Hatschek: Surface Tension and Surface Energy, 1923.

let us assume that there are only 20,000,000 units per c.c. in the original. In the 10^{-1} tube there would be 2,000,000 units per c.c., in the 10^{-2} , 200,000, in the 10^{-3} , 20,000, in the 10^{-4} , 2,000, in the 10^{-5} , 200, and in the 10^{-6} , 20. We are constantly decreasing the number of units handled, and thus putting more and more confidence in the laws of probability as applied to a small number of cases. In the 10^{-6} tube, we have 20 units in 10 c.c. of thoroughly mixed liquid. We remove 1 c.c. for the 10^{-7} tube. Perhaps we secure the most probable number of units, namely 2. It is not inconceivable that we secure only 1, or as many as 5. The 10^{-7} tube, then, will contain from 1 to 5 units. If there is 1, in taking 1 c.c. for the 10^{-8} tube once in every 10 times we carried out the procedure, we should secure 1 unit for the 10^{-8} tube; the probability that it happens is 1:10. If 5 units are present in the 10^{-7} tube, the probability is 5:10 (more exactly, 715:2002) of carrying at least 1 unit to the 10^{-8} tube, and for that matter the probability is not small for carrying 2 units to the 10^{-8} tube, whence there would be approximately a 2:10 chance of carrying the bacteriophage over the 10^{-9} tube. And, looking back, had there been 1 unit in the 10^{-7} tube, and had it been carried to the 10^{-8} tube by the tenth chance, the 10^{-7} tube would have been left with no bacteriophage units, and would have grown following inoculation, whereas the 10^{-8} tube would not have done so. We have observed this apparently paradoxical error in an "inhibition-titration" on several occasions, not due to the accidental contamination of organisms not sensitive to the lytic action of the bacteriophage. Thus, even by conservative estimate, our results have suggested merely that the bacteriophage concentration sought is between 10,000,000 and 1,000,000,000 per c.c., and the difference is too great to be of more than rough quantitative value.

Although there can be no gross difference between enumeration of areas by means of slants and by means of plates as procedures for giving agar surfaces, there are reasons for favoring the plate. In our preliminary experimentation, it was brought out that it is difficult to secure a dilution of bacteriophage which will give a number of areas easily counted on a slant. This is not true of the plate. Because of the difficulty in accurately measuring an amount of liquid deposited on an agar slant, the plate becomes superior. Furthermore, the conditions on all parts of the surface of an agar slant are not uniform, whereas a plate gives uniform conditions. This is particularly true of the moisture, which, in an agar slant, unless precautions are taken, distributes itself according to gravity. The top of the slant will be dry, giving small areas, whereas the moisture in the lower part of the slant may be so great as to cause slurred areas, or areas which have run together. Finally, we found it necessary to take a number of special precautions with an agar slant with a view to counteracting the foregoing faults, and in order to give comparable results with slant and plate methods of enumeration.

There is a further important point regarding the measurement of bacteriophage concentration, based on the fact that the two essential factors, the organisms and the bacteriophage, are mutually interactive. On exposure to organisms, the bacteriophage increases. It is hence imperative that the bacteriophage and the organisms be kept separate until such time as they may be fixed in space, and the bacteriophage, by means of organisms, forced to show its presence. In an inhibition-titration series, the tubes must not be inoculated before the serial dilution is made. In an area enumeration, the organisms should not be mixed with bacteriophage previous to inoculation to an agar surface, at least for any length of time.

Having developed in their broader aspects some of the factors involved in quantitative measurement, it becomes necessary to investigate some of the more specific questions involved.

The bacteriophage under investigation was an original d'Herelle type sent by him to Dr. F. G. Novy in 1921. The original activity of this bacteriophage was against *Eberthella dysenteriae* (Shiga), *Escherichia coli*, and *Salmonella paratyphi* (beta type), and subsequent enhancement of its activity was effected against the first named.

The question of what strain of *Eberthella dysenteriae* (Shiga) to use in the study of the phenomena was important. Although *Eberthella dysenteriae* (Shiga) is supposedly a true bacterial species, and as such should be similar in its receptivity to the bacteriophage regardless of the source of the bacterial strains, it became evident at the outset that no such assumption was safe. A study of the character of the areas produced by 3 different specimens of the d'Herelle bacteriophage, all from the same stated source but having received different treatment and being of different age, revealed not only a difference in the type of area against a single strain of *Eberthella dysenteriae* (Shiga), but the same bacteriophage specimen gave different types of areas and to some extent different numbers of areas with different strains of the Shiga dysentery bacillus. Three strains were used; the original Shiga strain from the Japanese epidemic, a Pasteur Institute strain, and a local strain, all true to type as judged by the ordinary biologic and biochemical tests. The types of areas were definitely divided into 2 classes, a large and small area, respectively, 2 to 5 mm. in diameter, and 0.5 to 1.0 mm. in diameter. One specimen of bacteriophage gave only the small type of area with any strain used. Among all the strains of the dysentery bacillus, the local strain proved the most sensitive, and very consistent in its activity with the specimen of bacteriophage giving only small areas. The consistency of interaction between these two variables, and the fact that we were unable by any means tried to demonstrate a greater bacteriophage concentration in any tube of this bacteriophage than could be demonstrated with this strain of *Eberthella dysenteriae* (Shiga) led us to employ it for subsequent experimentation. The variation of activity of a given bacteriophage against different bacterial strains supposedly of the same species has been noted by Davison,¹⁴ Janzen and Wolff,¹⁵ Bruynoghe and Appelmans,¹⁶ Pico,¹⁷ and others. It is not uncommon to find that a bacteriophage is active against one bacterial strain to a marked degree, but with no apparent effect on another strain of the same species.

There must be a reasonable degree of permanence of bacteriophage concentration in a culturally sterile broth filtrate containing bacteriophage; or, failing this, there would be another variation to study, and the pursuance of the variables, bacteriophage and organisms would be greatly complicated. D'Herelle's work would indicate an extreme of permanence of bacteriophage concentration in broth over a considerable period of time. Experimental investigation with the particular bacteriophage of our work over a period of 54 days revealed no change in the concentration. The experiment was terminated by an accident at this point, but we have recently had occasion to examine bacteriophage sealed for a year with the result that the concentration appears only

¹⁴ Jour. Bacteriol., 1922, 7, p. 475.

¹⁵ Konink. Akad. v. Wetensch., 1922, 25, p. 31; abst., Bacteriol., 1923, 7, p. 167.

¹⁶ Compt. rend. Soc. de biol., 1922, 87, p. 96.

¹⁷ Ibid., 86, p. 1106

slightly reduced, together with a slight reduction in the activity of the bacteriophage unit as indicated by slightly smaller areas.

Bordet and Ciuca¹⁸ made intraperitoneal injections of *Escherichia coli* into a guinea-pig. Some hours later the peritoneal exudate was found to contain a principle lysogenic for *Escherichia coli*. From their experiments they concluded that, under the influence of a product secreted by the leukocytes, the bacteria undergo a "nutritive vitiation" resulting in lysis. But, since the leukocytic principle disappears in the course of successive transfers in vitro, without loss of the lysogenic power, they assume that the nutritive vitiation is hereditary.¹⁹ D'Herelle has variously contended that this hypothesis is "purement verbale." He adds a small amount of filtrate containing the lytic principle to a bacterial suspension, and brings about lysis. He then filters this lysed bacterial suspension through a filter impermeable to bacteria. A small amount of this filtrate is added to a second bacterial suspension, and lysis brought about. This is filtered, and the series carried ad infinitum. Where, says d'Herelle,²⁰ is there place in this process for the transmission of an acquired characteristic, since he has allowed no bacteria to enter a filtrate which will be lysed if they possess the hereditary qualification? And how, he adds, can one invoke heredity when there are no descendants? Since our bacteriophage represents an infinite dilution of the original bacteriophage brought about by a procedure similar to that described, it may be stated with certainty that our bacteriophage falls in line with this argument of d'Herelle's. Two other experiments were carried out by d'Herelle in support of his contention that the phenomenon is not due to hereditary factors set up within certain bacteria. In the first, he used a definite concentration of bacterial suspension and varying amounts of bacteriophage, noting the areas produced when fixed amounts of the mixtures were inoculated to agar slants. Were Bordet's hypothesis, implying a definite proportion of organisms capable of the nutritive vitiation, correct, an equal number of areas should result on all slants. D'Herelle, however, found that the number was proportional to the amount of bacteriophage represented. In the second experiment, he added fixed amounts of bacteriophage to bacterial suspensions of varying concentration. Bordet's hypothesis would demand that the number of areas be proportional to the bacterial concentration; d'Herelle found that the number of areas was the same throughout. To ascertain whether the experimental grounds of Bordet's hypothesis might be applicable to our bacteriophage and bacterial strain, although not applicable to the combination used by d'Herelle, we repeated each of these experiments three times, with the result that d'Herelle's observations were fully confirmed. Asheshov, working with the types of bacteriophage isolated by himself, found corresponding results. It is, therefore, not necessary to consider the number of bacteria used in area enumeration work.

In the course of our study of methods of enumeration, a "live" agar medium was developed consisting of a 4% beef infusion agar to which, in the melted state at 45 C., was added an equal volume of 10 to 18 hour infusion broth culture of the sensitive organism. It was hoped that this medium would develop colonies sufficiently close together to reach any bacteriophage present, which would then demonstrate itself by clearing the agar at that point. Neither the addition of the bacteriophage to the melted agar, nor the pouring of the "live"

¹⁸ Compt. rend. Soc. de biol., 1920, 83, p. 1293.

¹⁹ Bordet and Ciuca: Compt. rend. Soc. de biol., 1920, 83, p. 1296.

²⁰ Rev. de Path. Comp. et d'Hyg. Gen., Oct. 5, 1923, 238.

agar and subsequent inoculation of the bacteriophage to the surface of the plate gave satisfactory results. Although each colony was estimated to have a working volume of only 20 to 30 microns cubed of agar, it was found that the areas produced in this manner did not account for all the bacteriophage units present; there were apparently some bacteriophage units not reached by bacterial cells in such a manner as to form visible areas. The use of indicator stains did not add to the value of a medium for enumeration. A 30% "live" gelatin treated similarly to the "live" agar failed completely; other authors have found gelatin unsuitable for bacteriophage work.²¹

The final procedure adopted for the enumeration of bacteriophage units, or the measurement of bacteriophage concentration, was as follows. The culture medium was a beef infusion 2% agar of P_H 7.4. Plates were poured, using 10 to 12 c.c. of agar, and dried, either for 24 hours or longer inverted, at room temperature, or at 37 C., for a shorter period of time, preferably not less than 10 hours. A proper degree of moisture was found to be essential, as an excess results in washed noncircular areas (plate 2, fig. 1), and too little moisture is not only undesirable for good growth of the bacteria, but also results in minute areas, so that one cannot be sure that all areas which may be present are apparent. To the properly dried, sterile plate was inoculated 3 drops of a rejuvenated beef infusion broth culture of *Eberthella dysenteriae* (Shiga) from 6 to 16 hours old, and spread with a glass rod. Since the number of areas produced by a given amount of bacteriophage is not affected by the number of organisms present, the amount of inoculum is not important; provided the culture be rejuvenated by frequent transfer and the culture from which the plate is made be still in, or near, the logarithmic phase of growth. D'Herelle has pointed out the necessity for the use of young cells for lysis by the bacteriophage, a point which we have checked. Calculation shows that at least 4 frequent transfers, at least twice a day, should be followed to eliminate any organisms irregular in their growth and not capable of keeping up with the regular logarithmic increase in concentration. The plate inoculated with organisms was incubated for from 2.5 to 5 hours at 37 C. for the dual purpose of drying and of allowing development of the culture, it being necessary to have a solid growth as a background for the bacteriophage areas. The plate was then ready for inoculation with the bacteriophage. This was made by adding a known, carefully measured amount of the suspension to be measured in the proper dilution to secure approximately a hundred areas to the surface of the plate. The amount should be not less than 0.05 c.c., and not more than 0.10 c.c.: less than 0.05 c.c. is difficult to spread properly, and more than 0.10 c.c. introduces too much moisture. The liquid is spread with a sterile glass rod to all but the extreme edges at two opposite points, these serving to touch off the rod before finally removing it. The spreading can be done with several rapid sweeping motions; it is not necessary to rub it in. The minute colonies are spread with the bacteriophage, so that following incubation there is a solid mass of culture surface in which the bacteriophage units may form lysed areas (plate 1). By this procedure, the bacteriophage unit is immediately fixed at a point where it must demonstrate itself, the conditions are uniform, and, of great importance, the organisms and bacteriophage are separated and have no opportunity to interact in any way before they are individually fixed at definite points. Incubation of the plates appeared to be reasonably unimportant; the usual procedure was to incubate at 37 C. overnight. Longer incubation, either at

²¹ Nakamura: Wien. klin. Wchnschr., 1923, 36, p. 86.

37 C. or at room temperature, caused neither an overgrowth of lysed areas nor the development of previously unseen areas.

The inhibition-titration method of estimating bacteriophage concentration is useful, provided the possible fallacies of the method are considered. In the first place, it forms a convenient method for making approximations where such information would be of use. In the second place, when dilutions of bacteriophage are made in broth for the purpose of securing the desired number of areas on a plate, several higher dilutions may easily be made, and the series of dilutions be inoculated with young rejuvenated culture, thus giving an inhibition-titration as a rough control for the results given by the plate.

The Variables: Bacteriophage and Organisms.—Normal Multiplication of *Eberthella dysenteriae* (Shiga): The normal rate in beef infusion broth is of interest not only here where it is essential as a standard whereby comparison may be made on the rate as influenced by the bacteriophage, but also perhaps because of the widespread use of this organism in bacteriophage work.

It has been shown by Chesney,²² Sherman,²³ and others that there need be no lag period in the growth of young cultures, provided the inoculation is made from the culture still in the logarithmic phase of growth into a similar medium at incubator temperature. If, with this precaution, we inoculate N organisms per c.c. into a medium, and the organism divides by binary fission every t hours, then at the end of any time T , taken in hours from the time of inoculation, the bacteria per c.c. considered as concentration C is

$$C = N 2^{T/t}$$

Thus, if we inoculate 2 organisms per c.c. ($=N$) multiplying every half hour ($=t$), then at the end of 2 hours ($=T$) there should be

$$\begin{aligned} C &= 2 \times 2^{2/0.5} \\ &= 32 \text{ organisms per c.c.} \end{aligned}$$

If $C = N 2^{T/t}$, then $\log C = \log N + T/t \log 2$, in which C and T are variables, and T plotted against $\log C$ is a straight line. $(\log 2)/t$ is the slope, whence t is easily determined.

The results of a number of experiments in which the increase in bacterial concentration was followed by plating methods are given in a chart in a paper by the author.²⁴ With *Eberthella dysenteriae* (Shiga) plating methods were found to be adequate in studying the logarithmic phase of growth. The cells exist singly in broth culture, and there is evidence that cells in the dividing state, that is, actively reproducing, grow uniformly. Averaging the figures for t , the time of binary fission, as calculated from the slope of the curve in each experiment, the result

²² Jour. Exper. Med., 1916, 24, p. 387.

²³ Sherman and Albus: Proc. Soc. Am. Bacteriol., abstr. Bacteriol., 1924, 8, p. 6.

²⁴ Jour. Infect. Dis., 1924, 35, p. 532.

indicated a division every 31.3 minutes. In the experiments shown, which were not selected especially for this purpose, the range of binary fission was between 29.4 and 34.0 minutes. The results pertain to the multiplication of *Eberthella dysenteriae* (Shiga) in beef infusion broth of P_H 7.4 at 37 C.

Organism Multiplication as Influenced by the Bacteriophage: How, then, is the normal rate of development of this organism influenced by the bacteriophage actively lytic for the strain? Since the ultimate bacterial concentration is zero, there must be a reduction in bacterial concentration from even the small number of the original inoculation. In order to throw light on this question, the following experiment was performed:

Data are given from 4 typical experiments, tabulated as A, B, C, and D. In each case, plating methods were applied in order to follow the progress of organisms growing in broth which contained bacteriophage. Known dilutions of bacteriophage were made in beef infusion broth, so that dilution 1 of experiment A represented a bacteriophage dilution of 10^{-5} of a concentrated bacteriophage filtrate; dilution 2 of this experiment, 2×10^{-6} of the same bacteriophage, and dilution 3, 10^{-6} of the same bacteriophage. The bacteriophage dilution of experiment B was 10^{-5} of a weaker filtrate than used in experiment A; in experiment C, the dilution was 4×10^{-3} ; in experiment D, it was 1:300. The bacteriophage concentrations represented were:

Experiment A, Dil.1	2,200 units per c.c.
Dil.2	440 units per c.c.
Dil.3	220 units per c.c.
Experiment B	100 units per c.c.
Experiment C	18,000 units per c.c.
Experiment D	580,000 units per c.c.

These dilutions of bacteriophage, together with a control broth containing no bacteriophage, were incubated to acquire a temperature of 37 C. Inoculations with *Eberthella dysenteriae* (Shiga) were made by adding known amounts of rejuvenated broth culture at 37 C. while still in its logarithmic phase of growth, so that, as far as possible, the control at least would continue to show the normal logarithmic rate of increase of bacterial concentration. The suspensions were incubated at 37 C. continuously for 12 hours, except for momentary removal at intervals for sampling and plating to determine the bacterial concentration. The results of these tests are shown numerically in table 1, and graphically represented in chart 1, in which the logarithm of the bacterial concentration is plotted against the time interval between inoculation and the time this concentration was determined.

A broth containing bacteriophage will allow a continuance of the logarithmic phase of growth of culture inoculated into it, under the proper conditions, for a greater or lesser period of time, after which lysis occurs rapidly.

From the results presented in table 1 and in chart 1, it appears that the development of organisms in the presence of bacteriophage follows a normal, logarithmic progression to a marked degree (considering that the bacteriophage might be expected to be active, to at least some degree, from the start) before there occurs a phase of rapid lysis. The age of

TABLE 1
GROWTH OF CULTURE EXPOSED TO BACTERIOPHAGE

Ex- peri- ment	Age of Culture in Hours	Dilution 1 of Bacteriophage		Dilution 2 of Bacteriophage		Dilution 3 of Bacteriophage		Control (No Bacteriophage)	
		Bacteria per C c.	Log	Bacteria per C c.	Log	Bacteria per C c.	Log	Bacteria per C c.	Log
A	0.00	1,206	3.08	1,206	3.08	1,206	3.08	1,206	3.08
	1.67	1,960	3.29	2,510	3.40	2,300	3.36	2,410	3.38
	3.50	12,382	4.09	20,600	4.31	21,400	4.33	—	—
	7.00	1,481,000	6.17	1,497,000	6.18	1,960,000	6.29	2,000,000	6.30
	12.00	10,740	4.03	200	2.30	3,900	3.59	274,000,000	8.44
B	0.00	1,413	3.15					220	2.34
	2.00	2,640	3.42					3,420	3.53
	4.00	36,900	4.57					36,500	4.56
	6.00	630,000	5.80					770,000	5.89
	8.00	10,700,000	7.03					18,700,000	7.27
	10.00	<1,000	3.00					300,000,000	8.48
	11.00	<100	2.00					—	—
	12.00	40	1.60					581,000,000	8.76
C	0.17	—	—					1,680	3.23
	0.75	1,940	3.29					—	—
	3.00	18,600	4.27					20,100	4.30
	5.50	571,000	5.76					627,000	5.80
	7.00	< or = 100,000	5.00					—	—
	8.00	< or = 1,000	3.00					—	—
	9.00	< or = 30	1.43					98,000,000	7.99
D	0.00	1,800	3.26						
	2.50	19,000	4.28						
	3.40	50,600	4.70						
	5.00	56,000	4.75						
	6.00	146	2.16						

the culture growing in the presence of the bacteriophage at the time when the phase of rapid lysis begins is not constant; nor, corollary to that, is the organism concentration constant at this point where rapid lysis begins. It may be concluded only that the progress of the bacterial concentration is dependent on the original concentrations of bacteria and of bacteriophage. It will be shown later that this is reasonable, and the manner of this dependence will be discussed.

It is first necessary to take up a study of the change in bacteriophage concentration during the interval in which the bacterial concentration changed in the manner just studied.

Bacteriophage Concentration as Influenced by the Organisms: During the time that the bacterial concentration is being influenced by the presence of bacteriophage, there must be a change in bacteriophage

concentration, for we know that a minute amount of bacteriophage may be introduced into a bacterial suspension, and after time has been allowed for lysis, the liquid will contain a high concentration of bacteriophage. The following experiment was performed to give some data on this point:

Dilutions of a potent bacteriophage were made in beef infusion broth, having the concentrations noted below in table 2. The suspensions were set in the incubator to acquire a temperature of 37 C., after which they were inoculated lightly with infusion broth culture at 37 C., rejuvenated so as to be

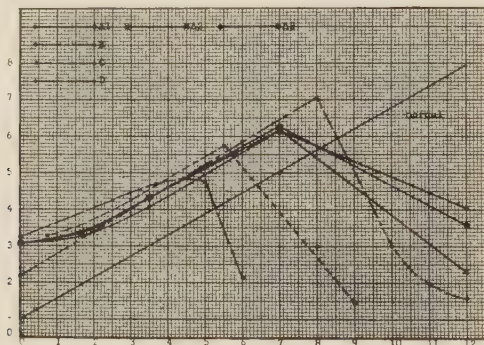


Chart 1.—Influence of bacteriophage on *Eberthella dysenteriae* (Shiga). The figures on the ordinate in this chart and in chart 2 indicate log bacteria per c.c.; the figures on the abscissa, the time in hours.

multiplying at the normal logarithmic rate. The inoculated suspensions were held at 37 C. throughout the experiment to determine the bacteriophage concentration, being removed from the incubator only to make dilutions and plates by the standard method for the enumeration of areas.

The results of two typical experiments, called A and B, are given in table 2, and graphically represented in chart 2, in which the logarithms of the bacteriophage concentrations are plotted against the age of the suspension following inoculation. The bacterial concentration is also represented over a synchronous period in order to show the relationship.

As the general trend of the bacterial concentration is to decrease, the general trend of the bacteriophage concentrations under the same conditions is to increase; and the period of increase is more or less synchronous with the decrease in bacterial concentration.

From the data presented, we observe that as the bacteria have been shown to follow for a certain length of time the normal logarithmic rate of multiplication, the bacteriophage concentration over approximately the same length of time is stable. Following this, there occurs what might be called a definite phase of rapid lysis of the bacteria and a

definite phase of rapid increase of the bacteriophage concentration. The ultimate bacterial concentration is zero, and the ultimate bacteriophage concentration appears to reach a maximum at approximately 500,000,000 units per c c.

A theory might be formulated as an explanation of the occurrence of a phase of rapid bacterial lysis and of rapid increase in bacteriophage concentration to the effect that cultures of a certain age represent a definite set of conditions as regards age of cells, presence of enzymatic action, or other unspecified conditions pertinent to a culture of a certain

TABLE 2
BACTERIOPHAGE INCREASE WITH LYSIS OF BACTERIA

Experiment	Time at Which Concentration Was Measured, Hours	Bacteriophage per C c.	Logarithm of Bacteriophage per C c.
A	0.00	18,000	4.26
	1.22	18,900	4.28
	3.33	13,858	4.14
	5.93	2,086,920	6.32
	7.38	99,262,800	8.00
	8.47	58,836,000	7.77
	9.58	129,870,000	8.11
B	0.00	580,000	5.76
	2.50	548,000	5.74
	3.67	2,548,000	6.41
	5.00	28,200,000	7.45
	6.00	113,500,000	8.06

age. There has been a tendency to explain some of the bacteriophage phenomena on such a basis—that of cellular condition as a function of time. We cannot believe, however, that the cellular condition is any but a continuous function of the age of the culture—that there is no sudden change of the conditions of the cells and their environment. On this basis there would be no evidence of conditions favoring a more or less sudden stimulation of lytic action. This should more especially appear logical since we are dealing as much as possible with organisms in their logarithmic phase of growth, the organisms multiplying in normal geometric progression with no evidence of any inhibitory agent or cellular eccentricity.

It would appear more logical perhaps to offer as an explanation of the phase of rapid lysis of bacteria, and of rapid increase in bacteriophage concentration, a theory based on the actual physical proximity of the

bacterial cells and the bacteriophage units; to say that the phenomena are due to the dispersion of the respective units.

The period of time elapsing between the inoculation of the bacteriophage dilution and the phase of rapid lysis of the bacteria decreases as the bacteriophage concentration of the original dilution increases. For example, from chart 1 it is apparent that the intervals between inoculation and the beginning of rapid lysis were respectively 5.0, 5.5, 7.0, and 8.0 hours; the corresponding original bacteriophage concentrations were 580,000 units per c c., 18,000 units per c c., 2,200 units per c c., and 100 units per c c., respectively. If the original bacteriophage concentration was 2,200 units per c c., a rapid drop in bacterial concentration originated

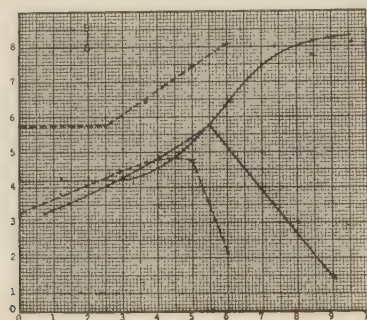


Chart 2.—Rate of increase of bacteriophage.

at or about the point where the bacterial concentration was 10,000,000 organisms per c c. When the bacteriophage concentration was originally greater, as 18,000 units per c c., the phase of rapid lysis began when the bacterial concentration had reached only about 600,000 per c c.

In a suspension containing a few organisms per c c. and a very few bacteriophage units per c c., under stable conditions which do not exist, the elements would in time probably get together and bring about lysis. However, the young culture under such conditions in reality proceeds to multiply apparently normally. The bacteriophage would seem to lie dormant until the organisms reach a certain proximity brought about by the increased bacterial concentration. At that time lysis begins with more than transient rapidity, and although the organisms constantly become fewer and thus more dispersed, the lysis has increased the concentration of bacteriophage units; hence the proximal relationship of the reacting

elements is not so much altered, and lysis proceeds to completion. This view of the matter puts the time element of lysis as a function of the dispersion of the two elements.

One might assume that if y were the concentration of bacteriophage units and x the concentration of organisms, that $x + y$ need only reach a certain figure to bring about rapid and complete lysis. If the organisms were present in a concentration of 10,000,000 per c.c. and the bacteriophage were present in a concentration of y in one case at the beginning of rapid lysis, then in another case in which the bacterial concentration was only 600,000 per c.c., the bacteriophage concentration might be assumed to be close to $y + 9,400,000$ per c.c. However, it is probable that the difference in size between the bacterial cell and the bacteriophage unit—which it is safe to say is considerable—enters into the matter, for an increase in bacterial concentration means a much larger surface for forming contacts than would a corresponding increase in the number of bacteriophage units. Examination of the data reveals no such simple rule as when $x + y = k$ rapid lysis occurs. On the other hand, it is apparent from chart 2 that although the organisms inoculated to the bacteriophage suspensions were nearly the same in both A and B, the period of rapid lysis of bacteria and increase in bacteriophage concentration began in a much shorter time when the original bacteriophage concentration was greater.

Maximum Concentration of Bacteriophage in Broth: Will the addition of n organisms to b bacteriophage units produce a final bacteriophage suspension containing k bacteriophage units? We should certainly be justified in predicting that, provided n and b were not too large, no constant number of bacteriophage units would result, for neither the bacteria nor the bacteriophage remain fixed; although lysis of one bacterial cell brought about by one bacteriophage unit may result in 15 or 20 bacteriophage units after lysis, we have seen that the addition of n organisms to b bacteriophage units results actually in the lysis of a much larger number of cells. Furthermore, the bacteriophage concentration in a tube of broth in which lysis has occurred seems to be more or less the same, whether the initial concentration of either bacteria or bacteriophage, or both, were high or low. Consequently, we might expect no great increase in bacteriophage concentration if b were very high. What actually happens under such experimental conditions is shown in the following tests:

Into a large sterile tube were placed 24 c.c. of a potent bacteriophage filtrate containing 226,000,000 units per c.c. To this was added 1 c.c. of rejuvenated broth culture, and lysis allowed to proceed at 37 C. for 24 hours, or, strictly speaking, beyond the point of lysis. Five c.c. were removed, and the bacteriophage concentration determined by the enumeration of areas. After standing several days at room temperature, the bacteriophage concentration was again measured, further organisms added, and lysis again brought about at 37 C. over a period of 24 hours. The bacteriophage concentration was measured, the tube allowed to stand several days at room temperature, and the operation repeated for a third time. These are tabulated in table 3 as "feeding" A, B and C. Following the third "feeding," the bacteriophage concentration was determined at 5 and 7 days as additional checking. The organisms added were counted by plating methods, so that the exact number might be known.

The results are shown in table 3.

TABLE 3
MAXIMUM CONCENTRATION OF BACTERIOPHAGE

"Feeding"	Date	Bacteria Added	Measurement of Concentration Before or After Lysis	Bacteriophage Units per C c.
A	11/28/23	869,500 in 25 c.c.	Before	226×10^6
	11/29/23		After	162×10^6
B	12/ 3/23	10,250,000 in 20 c.c.	Before	50×10^6
	12/ 4/23		After	145×10^6
C	12/ 6/23	300,000 in 15 c.c.	Before	$2,358 \times 10^6$
	12/ 7/23		After	221×10^6
(D)	12/11/23	None	After	$5,824 \times 10^6$
	12/13/23		After	$5,360 \times 10^6$

There was a gradual development of a flocculent debris in the bottom of the tube after the C feeding, in part, at least, due to the development of a resistant, or R, strain, as shown by the microscopic entity of the cells, and by the definite increase in the flocculent precipitate for a period of a week following the feeding C. Continued attempt at culturing the strain resulted only in sterile slants and plates.

From these data one observes that after a certain maximum concentration is reached, bacteria may be lysed without appreciably increasing the bacteriophage concentration. It is also apparent that there appears to be a super-concentrated bacteriophage coincident with the development of the resistant strain. This, as will be brought out in connection with a discussion of the resistant strain, is consistent with the properties of such a strain.

Corollary to the above experiment the following experiment, which was repeated a number of times, is pertinent.

An inhibition-titration was made on a potent bacteriophage. Dilutions up to and including 10^{-8} of this bacteriophage gave no growth following inoculation with rejuvenated culture, and incubation at 37 C. Dilutions of 10^{-9} or higher gave normal growth. After 48 hours of incubation of the inoculated dilutions of this bacteriophage, the dilutions themselves were titrated by a similar inhibition-titration.

It was consistently found that the maximum dilution giving complete inhibition was the same, whether the original tube represented a 10^{-1} dilution of the original bacteriophage, or any higher dilution giving inhibition and lysis.

It is logical to find demonstrated a maximum concentration of bacteriophage in broth, for if there resulted an infinite number of liberated bacteriophage units, even though lysis may continue to occur, chemical laws would require that such infinite concentration result in the separation of bacteriophage in its pure state. If we assume that the bacteriophage unit is a sphere 20 millimicrons in diameter, the maximum concentration is only 0.0000000009% actual bacteriophage material, whatever that may be. This maximum concentration does not take into consideration the concentration in or on a lysed area of bacterial growth on agar when a continuity of bacteriophage units might logically occur in much the same way as the continuity of bacterial cells grown on solid mediums.

Cellular Absorption of Bacteriophage.—There must inevitably occur either absorption or adsorption of the bacteriophage by living bacterial cells against which the bacteriophage is active, under the proper conditions. In order to bring about lysis, there must be some sort of physical contact between the bacteriophage unit and the bacterial cell. Even a theory based on an electrical field of force would not harmonize with the activity within the bacterial cell having a diameter of several microns, due to a force originating at any distance greater than that from the center of the cell to the outer cell wall. D'Herelle postulates the entry of a bacteriophage into a cell, multiplication there, and subsequent liberation of the units following lysis. Adsorption or absorption by dead bacterial cells has been demonstrated,²⁵ as well as adsorption by colloidal metals, serum, and charcoal.²⁶ We have attempted the study of absorption by living and dead homologous bacteria, by heterologous bacteria, and by the resistant or R strain of homologous bacteria.

Absorption by Living Homologous Bacteria: The following experiment gives some insight into the rate of absorption by living homologous culture, in spite of the fact that such experimentation requires the mixing of bacteria and bacteriophage in liquid suspension, thereby not fixing the two elements as constants on which to base interpretation.

²⁵ Jaumain and Meuleman: *Compt. rend. Soc. de biol.*, 1922, 87, p. 364.

²⁶ De Necker: *Ibid.*

A 16.5 hour culture of *Eberthella dysenteriae* (Shiga) in 40 c.c. of beef infusion broth was used. Ten c.c. of bacteriophage of a concentration of 174,300,000 units per c.c. was added, making 34,860,000 units per c.c. in the mixed suspension. The experiment was carried out at room temperature. The suspensions were filtered, 15 and 30 minutes, respectively, following the addition of the bacteriophage, enumeration of areas being made both before and after filtration in each case. The results are shown in the following tabulation:

Time	Bacteriophage per C.c.
0.00 hrs.	34.86×10^6
0.25 hrs., unfiltered	22.90×10^6
0.25 hrs., filtered	3.83×10^6
0.50 hrs., unfiltered	30.40×10^6
0.50 hrs., filtered	3.69×10^6

There appears to be a definite absorption of the bacteriophage by the bacteria, as well as, probably, by the filter in this experiment.

Bearing on the same point is the following experiment.

Two flasks containing 150 c.c. each of beef infusion broth were incubated at 37 C. to acquire that temperature. One was inoculated with young rejuvenated broth culture of *Eberthella dysenteriae* (Shiga), and allowed to develop to an approximate turbidity of 100,000,000 organisms per c.c.—i. e., not beyond the logarithmic phase of growth. To the control flask not containing bacteria was added a small measured amount of bacteriophage. The flask was shaken and filtered to saturate the filter with bacteriophage and to secure a control. Filtration was performed at 37 C. The culture was then mixed with a similar amount of bacteriophage, and held at 37 C., with filtrations made at 0, 15, 30, 45, and 60 minutes. Filtrate remaining in the candle and passing into the subsequent filtrate was taken care of by calculation as well as possible. The results were as follows:

Subject	Bacteriophage per C.c.
Unfiltered control	1,240,000
Filtered control	1,150,000
0' filtrate	1,050,000
15' filtrate	462,000
30' filtrate	810,000
45' filtrate	11,300,000
60' filtrate	25,400,000

There appears to be a marked absorption during the first 15 minutes, followed undoubtedly by further absorption, the results of which are masked by the natural increase in bacteriophage following lysis.

In these data there is apparent corroboration of the absorption of the bacteriophage by the bacteria during the initial stages of the lytic phenomena. Equally apparent is the liberation from lysed cells of bacteriophage units which are, if one considers the bacteriophage a living agent, the offspring of the absorbed units—all within less than 45 min-

utes. To say more than this would not be justifiable, for we have no means of ascertaining from the data presented, at just what period each bacteriophage unit was absorbed by a cell. Although the period between ingestion or absorption and the expulsion of the multiplied bacteriophage units should, under similar conditions, be nearly the same, the results gave only average figures of a large number of individual reactions, presumably starting at different times. In broad terms, we may say that during the first 15 minutes, under the conditions of the experiment, there occurs some sort of absorption of the bacteriophage units by the cells, the extent of absorption depending on the concentration of both elements. During the interval between 15 and 30 minutes some of the absorbed bacteriophage units, presumably those first coming in contact with the cells and thus first going through the multiplying phase, have acted on the bacterial cell, with subsequent lysis and liberation of more bacteriophage units than were absorbed. During the interval between 30 and 45 minutes most of the first bacteriophage units absorbed have passed through one cycle, and beyond much doubt many of them have been reabsorbed by bacteria. It is not improbable that some units have been through one cycle, been reabsorbed, and passed through another complete cycle at the end of 45 minutes. Beyond 45 minutes the results become confused, as speculation allows the presence of two or three cycles, with the probability that there are still some of the original bacteriophage units not yet through one complete cycle, since lysis had not occurred completely at the end of the experiment.

Absorption by Dead Homologous Bacteria: The following experiment was performed in order to throw some light on the rate of absorption by dead homologous bacteria, since we may assume from the work of previous authors that absorption should occur with such organisms.

A flask culture of *Eberthella dysenteriae* (Shiga) in 150 c.c. of beef infusion broth was subjected to a temperature of 62 C. for 15 minutes at such time as the bacterial concentration was approximately 100,000,000 per c.c. This time was allowed on account of the volume of liquid to be heated. Checks on the sterility demonstrated the absence of living cells. The cells remained in suspension without agglutination and without settling for 48 hours, when the experiment was performed. Duplicating the conditions of the absorption experiment with living cells, bacteriophage was added to a flask of sterile broth so as to have a concentration of 1,130,000 units per c.c. This was filtered at 37 C., making enumeration counts before filtration, and the first and second fractions of the filtrate. The same amount of bacteriophage was added to the killed

culture suspension at 37 C., and this was filtered at 0, 15, 30, 45 and 60 minutes. Filtrate remaining in the candle was accounted for by calculation. The results are shown in the following tabulation.

Subject	Bacteriophage per C c.
Original bacteriophage	1,130,000
1st fraction, control	690,000
2nd fraction, control	840,000
0' absorption filtrate	650,000
15' absorption filtrate	599,000
30' absorption filtrate	380,000
45' absorption filtrate	230,000
60' absorption filtrate	190,000

There is clearly indicated an absorption by dead homologous cells, progressive with time.

The results of the preceding experiment, clearly indicating absorption, do not permit an absolute statement that there is no increase in bacteriophage concentration from exposure to dead homologous bacteria. If, however, there is such an increase, it is certainly slight. The decrease in bacteriophage concentration in the first fraction of the control filtrate is no doubt due to adsorption by the filter. If this be true, the amount of bacteriophage adsorbed by the filter would depend on many factors, such as the porosity of the filter, finer filters presenting more surface than coarse ones, the H-ion concentration, the electrical charge of the micellae being directly concerned, and other physical factors. It has been demonstrated by Mudd²⁷ that serums when filtered lose some of their immune bodies, and methylene blue, for example, may be filtered out for some time before the surface, originally electronegative to the liquid, becomes electrically neutral or perhaps electropositive through saturation. On this basis, a demonstration of adsorption of bacteriophage by a filter implies that the units are electropositive to the filter surface in broth. These explanations suffice to explain adequately the presence of more bacteriophage per c. c. in the second fraction of the control filtrate than in the first, saturation having been approximately completed in the filtration of the first few c. c.

Absorption by Living Heterologous Bacteria: We may, for our present purpose, define heterologous bacteria as including all species except the normal sensitive *Eberthella dysenteriae* (Shiga). The following experiments were performed to ascertain the facts regarding the possible absorption by living cells of *Escherichia coli*, *Eberthella typhi*, *Eberthella paradysenteriae* (Flexner), *Eberthella dysenteriae*

²⁷ Am. Jour. of Physiol., 1923, 63, Feb.

(Shiga) (not the standard sensitive strain), and a resistant or R strain isolated from the homologous *Eberthella dysenteriae* (Shiga).

In testing for possible absorption by *Escherichia coli*, 12 hour flask cultures were allowed to develop in beef infusion broth following inoculation from a well rejuvenated culture. A small amount of bacteriophage was added to this culture at 37 C., so that the bacteriophage concentration was 800,000 per c. c. A control flask containing the same amount of broth as the culture flask, but no organisms, was used, keeping the flask at 37 C., and adding the bacteriophage to the same concentration. Similar amounts of culture—bacteriophage mixture and of control broth containing bacteriophage were filtered through similar filters. The absorption period was 15 minutes at 37 C. The results showed:

Subject	Bacteriophage per C.c.
Original unfiltered	800,000
Control filtered	714,000
Absorption filtrate	274,000

There thus appears to be a definite absorption of the bacteriophage by this strain of *Escherichia coli*.

In the case of absorption by *Eberthella typhi* (Eberth) the same technic was followed as in the test with *Escherichia coli*. The following results were obtained:

Subject	Bacteriophage per C.c.
Original unfiltered	287,000
Control filtered	256,000
Absorption filtrate	267,000

In this case there would seem to be a conclusive lack of absorption by this culture of the typhoid bacillus.

Application of the same technic to a strain of *Eberthella paradysenteriae* (Flexner) gave the following:

Subject	Bacteriophage per C.c.
Original unfiltered	287,000
Control filtered	256,000
Absorption filtrate	167,000

This culture apparently shows some absorption of the bacteriophage.

Similar technic was applied to a strain of *Eberthella dysenteriae* (Shiga), not the Shiga type against which the bacteriophage activity had been built up. This might be considered homologous absorption, except for the fact that at the time of the experiment the bacteriophage was only slightly active against this strain. The results follow:

Subject	Bacteriophage per C.c.
Original unfiltered	180,000
Control filtered	128,000
Absorption filtrate	210,000

The bacteriophage, being somewhat active against this strain, is undoubtedly absorbed to some extent. The fact that it is not apparent from the results is presumably due to the increase in bacteriophage units owing to lysis of some of the cells.

The absorption of the anti-Shiga bacteriophage was also attempted by the use of the resistant homologous strain of *Eberthella dysenteriae* (Shiga). The

technic of the previous tests was applied, with the one exception that the bacterial suspension was made by washing off agar slants with broth, for the reason that such resistant strains do not grow well in broth. The results were as follows:

Subject	Bacteriophage per C.c.
Original unfiltered	287,000
Control filtered	256,000
Absorption filtrate	6,000,000

It is apparent not only that no absorption occurs, but that the number of bacteriophage units in the "absorption filtrate" had actually increased many times. The result clearly indicates the presence of a large amount of bacteriophage closely correlated with and constantly present with an R strain. To demonstrate absorption would necessitate the removal of the bacteriophage thus carried along with the strain, and it is our belief that, could this actually be accomplished, a normal strain would result.

Summarizing as a group the experiments with absorption by heterologous but not distantly related bacterial strains, there appears to be no particular specificity of reaction as regards the ordinary bacteriologic grouping by serologic and biochemical reactions. From the serologic reactions, one would expect *Eberthella typhi* and *Eberthella paradysenteriae* (Flexner) to be as closely related to *Eberthella dysenteriae* (Shiga) as is *Escherichia coli*, although the results of the absorption tests might indicate the reverse. Judging by the adsorption by inanimate substances and by the lack of specificity of the bacteriophage in lysis of strains presumably identical, it is questionable whether specificity of this type can logically be expected.

There is, however, another type of specificity of absorption which might be considered—absorption by bacterial cells susceptible to the specific lytic action of the particular bacteriophage to a greater or less extent. Regarding the matter from the point of view of the bacteriophage, this would be specific absorption. The results of the preceding experiments do not indicate a specificity of this sort. Absorption by the R strain must be omitted from consideration, but the bacteriophage used was slightly active against *Eberthella dysenteriae* (Shiga) of this heterologous series, and was originally active against the strain of *Escherichia coli* used, although we could secure no signs of areas at the time of the absorption experiment. The absorption tests do not correlate with the bacteriophage activity in this particular series, in spite of the fact that specific absorption of this type is extremely probable.

Absorption or Adsorption: In the case of the living cell, the probability is that the focus of reaction is within the cell rather than at its surface. With the dead cell, however, we may very well have

adsorption. The only reason for this statement thus far apparent would be the analogy between possible adsorption by the dead cell and the definite adsorption by substances such as filter surfaces, colloidal metals, and the like. If the bacteriophage unit is absorbed by a dead cell, it should not be free to produce areas in living cultures on an agar plate, whereas if adsorption occurs merely at the surface of the dead cell, there should be active contact between the bacteriophage and living cells when opportunity is offered on an agar surface by the usual procedure for the formation of lytic areas. The following experiment is pertinent.

A tube of beef infusion broth, 20 cc., was inoculated with rejuvenated *Eberthella dysenteriae* (Shiga). When, following incubation at 37 C., the bacterial concentration had reached approximately 100,000,000 per cc., the organisms were killed by exposure to a temperature of 62 C. for 10 minutes. To this killed culture suspension, as well as to a control tube containing the same amount of broth but no organisms, was added a definite amount of bacteriophage. The bacteriophage concentration of the control was measured by plate enumeration of areas. The killed culture was exposed to the bacteriophage for 2 hours at 37 C., counts of the bacteriophage concentration being made at 15 minutes, 1 hour, and 2 hours (whether or not this really is a measurement of the bacteriophage concentration is, of course, the point of the experiment). The results follow:

Subject	Bacteriophage per C.c.
Control	12,000
Adsorbed 15'	10,300
Adsorbed 1 hr.	16,600
Adsorbed 2 hrs.....	17,400

It appears that the bacteriophage in the presence of killed bacterial cells, although previously demonstrated to be taken up by such cells, was still so situated as to come in contact with living cells inoculated to a plate for area enumeration.

Thus, if our reasoning is correct, adsorption of bacteriophage by dead cells would be indicated, and not absorption. We should not consider the apparent slight increase in bacteriophage concentration to be of any significance.

Diffusion of Bacteriophage.—Through Agar: It was observed in the course of experimentation that in several instances in which moist plates were used the culture had run underneath the agar and developed between it and the glass. In such plates, the surface growth might be completely lysed with no effect on the culture underneath. The diffusion of the bacteriophage in agar would seem to be limited to a distance less than the thickness of an agar plate. The following experiment was performed in order to demonstrate the presence or absence of diffusion in agar under controlled conditions.

Small sterile tubes of 4 mm. internal diameter were made and filled with "live" agar, that is, a 4% agar mixed in equal parts with a young broth culture. This was allowed to harden, and a drop of concentrated suspension of bacteriophage was dropped on the surface. The diffusion could be noted in the small tube by the clearing of the agar due to the lysis of the small closely associated colonies.

Diffusion took place to a depth of 1 mm. gradually over a period of 48 hours, beyond which, of course, there was no change, owing to the increased age of the bacteria. Partial anaerobic conditions might have had some slight influence. Various dyes under similar conditions diffused a centimeter or more in the same length of time.

Diffusion of the bacteriophage in agar is borne out by the work of Arnold.²⁸

Diffusion Through Parchmentized Membranes: The possible diffusion of bacteriophage through parchmentized membranes was studied by means of dialysis of a broth suspension of bacteriophage with distilled water.

Parchmentized thimbles 2x9 cm. were sealed to short glass tubes 2 cm. in external diameter by means of a rubber band and collodion. The tubes were not plugged with cotton, but were placed in larger tubes, which were plugged and sterilized in the autoclave. Distilled water dialysis was carried out at room temperature, about 23 C. Five wash waters were used in 2½ days, all wash waters being saved and measured for bacteriophage concentration by the usual plate method. The concentration of the original suspension was determined, and of the dialysate after the final washing. 12 c.c. of suspension were originally placed in each tube, resulting in about 14 c.c. in the final dialysate through osmosis; 14 c.c. of sterile distilled water were used for each washing. The results were as follows:

Subject	Bacteriophage per C c.
Original concentration	1,240,000
First dialysate	686,000
Second dialysate	754,000
1st wash water, 0-20 hrs.....	25,100
2nd wash water, 20-25 hrs.....	17,900
3rd wash water, 25-44 hrs.....	19,350
4th wash water, 44-49 hrs.....	21,500
5th wash water, 49-68 hrs.....	28,500

From these data one may conclude that the bacteriophage is diffusible through parchmentized membranes, and that diffusion occurs as a continuous function of time.

These data, besides presenting evidence of diffusion of the bacteriophage through parchmentized membranes, appear to show a considerable adsorption by the membrane, for all of the bacteriophage is not accounted for, and the only condition which would cause one to suspect destruction of the bacteriophage is exposure to distilled water. Although not impos-

²⁸ Jour. Lab. & Clin. Med., 1923, 8, p. 720.

sible, it is not probable in this experiment, for there was no tendency for the bacteriophage units to produce areas diminished in size, such as might indicate an intermediate step in diminished "virulence" between no effect as a result of exposure to distilled water and complete destruction. The total number of bacteriophage units originally present less the sum of the bacteriophage units of all the wash waters might be expected to equal the total number of bacteriophage units present in the dialysate. That this is not so is apparent from the following:

Original bacteriophage units: 12 c c. x 1,240,000 or.....	14,880,000
Dialysate bacteriophage units: 14 c c. x 700,000 or.....	9,800,000
Lost	5,080,000
Wash water 1: 376,000	
Wash water 2: 168,500	
Wash water 3: 290,250	
Wash water 4: 322,500	
Wash water 5: 427,500	
	1,584,750
Apparent loss unaccounted for.....	3,495,250

The discrepancy is marked. The figures used for volume of liquid are inexact, but liberal allowance was made where there was doubt, with a view to securing a minimum figure for the bacteriophage "unaccounted for." Thus the result, although it may be somewhat too small, is not too large.

Diffusion Through Collodion Membranes: Dialysis of a broth filtrate containing bacteriophage was carried out both with running tap water and with distilled water. The details of the experiments were as follows.

Tap water. Fat extraction thimbles 2 x 8 cm., made of hardened filter paper, were sealed to short glass tubes of 2 cm. external diameter by means of a rubber band and collodion. The thimbles were then plunged 5 times into collodion, allowing a minute to elapse between immersions, and placed in water. The glass tubes were then plugged with cotton, and sterilized in the autoclave. A broth filtrate suspension of bacteriophage was introduced into the sterile tube, the whole inserted in a beaker filled with tap water, and running tap water allowed to flow over it for 3 days. Bacteriophage concentrations before and after dialysis were determined by area enumeration, with the following results, shown for 2 duplicate tubes:

Subject	Bacteriophage per Cc.
Original concentration	4,040,000
Dialysate No. 1.....	1,760,000
Dialysate No. 2.....	1,360,000

The results would seem to indicate a definite diffusion of the bacteriophage through a heavy collodion membrane.

Distilled water. Thimbles were prepared in a manner similar to those used in the experiment with tap water, but with only two immersions in collodion, making a membrane of lesser thickness. These tubes were not plugged with cotton, but were placed within larger tubes, the larger tubes being plugged, and the whole sterilized in the autoclave. The bacteriophage filtrate in broth was introduced into the collodion thimbles in 10 c.c. amounts, and sterile distilled water (5 c.c.) into the larger tube. The bacteriophage used had a concentration of 24,200,000 units per c.c. Dialysis was allowed to proceed at room temperature. From the large tubes the first wash waters were removed after 4 hours and in 2 experiments found to contain 14,600 and 40,000 units per c.c., respectively.

Dialysis through thin collodion membranes was thus shown to occur, but further attempts to carry through the enumeration exactly were frustrated by the fact that the size of the areas became so small that it was impossible to say with any certainty how many were present on a plate. This diminution in the size of the bacteriophage area possesses special significance which will be referred to on a later page.

No attempt has been made to calculate the approximate size of the bacteriophage unit from these diffusion data. It is apparent that such units in their narrowest dimension must fall within the realm of colloidal micellae, that is, between 0.14 and 0.00016 microns, the former limits representing the limits of resolving power of the ordinary microscope, and the lower representing the size of the hydrogen molecule. We feel that any further analysis of the probable size from the foregoing diffusion data is not justifiable because of the great probability that adsorption is an important, unmeasured factor, and because our technic lacks the great and carefully checked precision that should precede estimation by this means.

Resistant Strains.—Certain strains of bacteria against which a bacteriophage is active may be exposed to the bacteriophage, under the proper conditions, and variant strains resistant to the lytic action of the bacteriophage isolated. D'Herelle described several procedures whereby this may be accomplished, both of which could be easily duplicated with our bacteriophage and strain of *Eberthella dysenteriae* (Shiga). One procedure involves the preparation of a plate as for the production of areas, and the addition of an excessive amount of bacteriophage, so that at least part of the growth is entirely cleared. On this cleared zone, there will appear in 24 to 48 hours, small, perfectly symmetrical colonies, granular, dry, and cohesive, as shown by the fact that they may be moved as an unbroken colony along the surface of the agar with a platinum needle. These colonies may be subcultured to agar, on which they will present a similar dry, granular, and very irregular growth (plate 2; fig. 3). It has been our experience that not more than 50% of such

subcultures are likely to develop, and that when once a subculture is secured, further transplantation is beset with difficulties, most strains having a tendency to run out entirely. Another procedure for the isolation of resistant strains involves excessive feeding of a bacteriophage with living, lysable cells, following which there occurs a flocculent débris in which the cells are intact, somewhat granular occasionally, usually more oval than the typical dysentery bacillus, and undoubtedly living in the presence of the bacteriophage and multiplying there, as is manifested by the marked increase in amount of flocculent débris. Isolation of this strain, however, is difficult.

The resistant strain presents no biochemical abnormalities. All sugar fermentations are normal, although very slow, owing to the slow rate of growth. Several strains carried over a period of a year lost no characteristics, nor did they gain new ones. During a month of that time transfers were made at least once, and often twice, daily, transferring always from the top of the slants where growth is ordinarily most regular, with no recurrence of the strains to normal growth. Broth cultures give a flocculent growth settling to the bottom of the tube, similar in every respect to the direct production of resistant strains in bacteriophage suspensions. They are capable of only several subcultures in broth, and are difficult to reisolate on agar. Serologic agglutination tests showed that a normal sensitive strain gave good agglutination both with a Mulford immune serum and with our local immune serum, whereas in no case did the resistant strains agglutinate with these serums.

The tendency toward autoagglutination of the resistant strains in broth caused us to look for a specific agglutinating substance. Filtration of both broth cultures and washings from agar slants, however, in no concentration used gave agglutination on incubation with a normal culture antigen.

The water of condensation of an agar slant culture of a resistant strain contains a considerable amount of bacteriophage. It was also found that a bacteria-free filtrate from a suspension of resistant organisms possessed a considerable concentration of bacteriophage units. There is ample evidence of the existence of the bacteriophage outside of the cells of an agar culture of the resistant strain. There is presumably in a resistant culture also a more or less variable amount of bacteriophage enclosed by the walls of various resistant cells. An attempt to wash off the external bacteriophage from the cells of a resistant culture suspended in salt solution, by means of successive centrifugings, taking off the

supernatant fluid each time and resuspending in salt solution, failed to separate the external and internal bacteriophage. The supernatant fluids of each of 4 successive centrifugings contained virtually the same amount of bacteriophage, and plating for enumeration of areas of the sediment resuspended to the original volume showed no appreciable reduction over a similar plating of the original suspension. Whether the results were due to an uncontrolled disintegration, or to the fact that continued resuspension of the sediment even with the most violent agitation did not give properly homogeneous results for washing, it is difficult to say. Homogeneous suspension of the bacteriophage from the concentration in which it is present on an agar slant is in itself perhaps parallel to suspending bacteria from such a surface growth—a complex physical problem. In this case, it is further complicated by the presence of the resistant organisms and their associated bacteriophage. A further attempt to locate the focus of the associated bacteriophage was made by heating a suspension of resistant culture at 60 C. for 10 minutes, killing the organisms but not killing the bacteriophage. Thus the absorbed bacteriophage in the resistant cells should be barred from the production of areas, whereas adsorbed or free bacteriophage would be free to form areas. A control suspension of bacteria-free bacteriophage was similarly heated. An average of a number of plates gave 18.5 areas for a certain volume of the unheated bacteria-free bacteriophage, and 20 areas for the same volume of heated bacteriophage; thus, the heating was not too great for the bacteriophage. Plating the unheated resistant strain suspension gave approximately 100 times as many areas as did a similar amount of the heated suspensions.

Thus, although we have no quantitative data on the exact relationship between free and absorbed bacteriophage in a resistant culture, there is good evidence that there is an abundance of each in such a culture. The relationship is, in our experience, too intimate for quantitative resolution except perhaps by very detailed micro-methods; for the marked irregularity of growth and the fact that disintegration of certain cells is bound to occur would indicate beyond much doubt that the free and absorbed bacteriophage are variables with a number of conditions, uncontrollable unless single cells might be used.

It is our belief that the resistant strain consists of normal cells exposed to the bacteriophage, but which, through unfavorable conditions, are never completely lysed. The resistant strains may be plated by streaking in high dilution, and from such plates regular normal colonies

may be picked, resulting in normal cultures, as well as irregular, typically resistant strains. Such resistant strains may be replated, and both strains reisolated. Again, the water of condensation of an agar slant culture of a resistant strain may be shown to possess a high concentration of bacteriophage units. The results of the experiment on absorption of bacteriophage by resistant cultures demonstrates the presence of a high concentration of bacteriophage with the dry growth on an agar slant. Subcultures in broth would seem to fail because of the carrying along of the multiplying bacteriophage, lysing all normal cells as they are produced, and leaving behind the slowly reproducing cells with an acquired resistance. The slow growth of the resistant strain would indicate that age is an important factor, for it is well established that an old cell is not especially well adapted to lysis by the bacteriophage. However, this would not seem to tell the whole story, for an old cell in the presence of bacteriophage must reproduce, even though slowly, thereby offering younger cells for lysis. We should thus picture the resistant strain as having adsorbed and absorbed bacteriophage, certain more or less normal cells, and a large proportion of old cells with an acquired resistance. Under proper conditions of isolation, such as on an agar surface or through separation in a liquid medium, certain cells are enabled to reproduce with sufficient rapidity to make them susceptible to lysis in broth, or to produce a normal strain on agar. Certain other cells are too closely associated with bacteriophage to become separated, and the slow development of a resistant colony may be viewed as a resistant cell dividing, producing two cells still possessing much the same relationship to the bacteriophage as the original cell and having the same acquired resistance, these cells reaching gradually the same conditions through aging as the original cell, whence four are produced, and so on.

This view of the typical resistant strain does not explain a variation in a susceptibility of different strains of bacteria, presumably of the same species, to the action of the bacteriophage. We made repeated efforts to adapt a bacteriophage isolated from a case of chronic colitis, and active against a strain of *Eberthella typhi* (Eberth), to lytic action against a biochemically and serologically identical Rawling's strain, without success. Adaptation of various types of bacteriophage from activity against one bacterial species has been reported in many instances, but in most cases it is not clear whether a single bacteriophage type or several mixed varieties are involved. The fact that some reports note

a loss of activity of the bacteriophage for the original culture in acquiring activity against another culture might seem to indicate association of several varieties of bacteriophage. The possibilities of associative action with mixed bacteriophages is not well worked out, but a review of the literature would indicate that as much care is necessary in using mixed types of bacteriophage as in working with mixed bacterial cultures; and the problems of associative action may be equally complicated in the two cases.

Lysed Culture as Antigen Material.—Reports in the literature reveal a lack of consistency of results as regards the specificity of the lysed cell protein, some supporting such specificity, others failing to demonstrate it.

Working with the anti-Eberthella typhi (Eberth) bacteriophage previously mentioned, we inoculated 3 guinea-pigs with lysed culture antigen, and 3 with ordinary killed culture antigen. Four injections were made at 3-day intervals, using 0.5 c.c. subcutaneously, 0.25 c.c. intravenously, and 0.5 and 0.5 c.c. subcutaneously, in their respective order. The lysed culture antigen was potent, having a bacteriophage concentration of 500,000,000 per c.c. The killed culture antigen had a similar concentration of killed bacterial cells. One week after the last injection, 2 c.c. of blood was removed from the heart of each animal, the serum taken off, and the antibody content determined by the following tests:

The agglutination titer of the serum of the 3 animals receiving the regular killed culture antigen was 1:640, as tested with a fresh homologous culture antigen. The agglutination titer of the 3 animals' serum receiving lysed culture antigen was 0 against a similar antigen. The serums of all 6 animals, as well as other antityphoid serums, were tested for the presence of specific precipitins for lysed Eberthella typhi (Eberth), but in no case could such precipitins be shown. We did not succeed in gaining sufficient virulence for the Eberthella typhi (Eberth) to determine, by means of protection experiments, the relative immunity acquired by animals inoculated with normal culture as compared with animals inoculated with lysed culture.

As a result of our experiments, we are inclined to view the lysed cell protein as lacking the specificity of the protein of the intact or mechanically disintegrated cell. This does not agree with the work of d'Herelle on barbonne, which seems to indicate specificity, or with other workers with various types of bacteriophage and other bacteria. The problem offers, however, so many varied angles that a further accumulation of data from all sources, with a variety of types of bacteriophage, bacterial strains, and serologic technics should be awaited.

DISCUSSION

This study of the bacteriophage of d'Herelle has not been undertaken with a view toward the solution of the question of the nature of the

lytic agent. Nevertheless, it has been impossible to observe various manifestations of the activity of the bacteriophage over a period of two years without gaining some impressions as to the nature of the bacteriophage activity.

There can be little doubt that the instigating agent of the lytic phenomena of the bacteriophage exists in a corpuscular state, as well separated and well organized units. The production of lysed areas in mass culture growth implies a localization at the focus of each area of some instigating agent in itself an entity, and it is, incidentally, difficult to conceive of any ionizable chemical substance possessing such a property. The presence of bacteriophage in a broth dilution of 10^{-9} of a filtrate of maximum concentration implies a colloidal suspension. A bacterial or colloidal suspension may be diluted greatly and subsequently shown to be present in the high dilution; a dilution of normal HCl to 10^{-7} is considered to be no more acid than the water in which it is contained. Under proper conditions, a dilution of a colloidal solution may be a mechanical procedure; a dilution of other solutions is not.

The production of "pie-shaped" or crescent-shaped colonies bears out the unit structure of the bacteriophage. We were invariably successful in producing these colonies by adding to melted agar at 45 C. a small amount of bacteriophage, slanting the agar, and inoculating the surface with culture in such a manner as to produce colonies rather than solid growth (plate 2; figs. 4 and 5). If a single bacillus is sufficiently close to a bacteriophage unit, no growth becomes apparent; but if a colony of bacteria is fairly started, and in spreading to an increasing diameter meets a bacteriophage unit, there occurs lysis on that side of the colony and subsequent inhibition to further growth in that direction. Inoculation of such a slant so as to produce heavy growth verifies the explanation, for areas are produced in such frequency as the number of crescent shaped colonies found would indicate. Colonies on such slants vary from large colonies with very small niches at the extreme edge, the original organisms or organism of the colony having been several millimeters from the nearest bacteriophage unit in the surface, to colonies with deep niches on three or more sides.

This method for the production of pie-shaped colonies affords a rough method for estimation of the size of the bacteriophage unit. Organisms to be lysed must come in contact with the bacteriophage units, and the organisms are spread only on the surface of the agar. If a definite number of bacteriophage units are placed in a definite quantity

of agar, the number of those units appearing in the surface is a function of the concentration of the units, their size, and the area exposed. This area, the number of units introduced, and the number of active units in the surface may all be determined. Calculation on this basis has given us results roughly between 10 and 100 millimicrons for the size of the bacteriophage unit. This procedure is not accurate in view of the complexity of the phenomena and the small size of the particles, and would not, unsupported, bear too close analysis. It is, however, of interest because of the fact that results thus given agree with those of other investigators,²⁹ who place the size in the neighborhood of 20 millimicrons in diameter. The matter is further complicated by the fact that we do not know whether the bacteriophage unit has a longer and shorter dimension, or whether it is spherical.

D'Herelle claims to have established a certain relationship between the potency of a bacteriophage filtrate and the number of ultramicroscopic granules of a uniform size as observed by actual ultramicroscopic examination. We have examined suspensions under the ultramicroscope and have seen the granules of uniform size, presumably the granules referred to. The observation would not lead me to make any more definite statements than those made by d'Herelle; and indeed the complex nature of the broth medium renders any definite statements of this nature of somewhat doubtful value.

The definite evidence in favor of a unity of structure, together with the oft-repeated argument based on the fact that lytic action is transmissible by repeated transfer of bacteriophage and exposure to organisms to what would be infinite dilution of the original bacteriophage filtrate, are, in my opinion, strong arguments in favor of the living nature of the agent. The fact that the bacteriophage gives evidence of assimilation of heterogeneous inanimate material into its own substance is, as mentioned in the introduction, strong evidence of the living nature. We have some evidence of the diminishing of the virulence or potency of the lytic activity, both by storage over long periods, and in one case with dialysis with distilled water. Variation of this sort is a property of living matter. The adaptation of a bacteriophage to a culture strain not that against which the lytic activity was built up would, if accepted, definitely favor a theory that the agent is living.

Our picture of the bacteriophage would, then, be an instigating agent of an organized, living, ultramicroscopic nature. We should picture this

²⁹ Prausnitz: *Centralbl. f. Bakteriol.*, I, O., 1922, 89, p. 187.

cellular unit as attacking a lysable bacterial cell and elaborating, either at the surface of the cell or within its walls, a substance which acts on the cell and brings about its lysis. Since there results coincident with lysis an increasing number of bacteriophage units, there is, be the agent living or inanimate, an increase at or in the cell. Such increase must be at the expense of the cell, unless anabolism is stimulated by contact with the cell and comes from the medium, which seems improbable. Because of quantitative considerations, on the basis of an elaboration of approximately 20 bacteriophage units, say 20 millimicrons cubed from one such unit within the cell, it is not logical to assume that anabolism alone is responsible for the lysis of the cell, and thus the postulate of the elaboration of a ferment, as perhaps a proteolytic enzyme, becomes an essential feature of a theory.

The data presented offer no clues as to the nature of the more intimate reaction between metabolic products of the bacteriophage, if it be living, and the bacterial cell. It is merely our purpose to state a theory compatible with the data, presenting our picture of the mechanism of the phenomena. The details of the nature of the lytic activity of the bacteriophage will, in the course of time, develop naturally from accumulated information, and to state any theory with undue vehemence at this stage of development is unwise and unnecessary.

SUMMARY

The purpose of the present work was the study of the phenomena of a d'Herelle type or strain of bacteriophage from a quantitative point of view, particularly as regards the mutual relationship between bacterial cells and bacteriophage. Studies of certain corollary features were also made from a quantitative point of view, specifically as regards the absorption of bacteriophage by bacterial cells, the diffusion of bacteriophage through agar and semipermeable membranes, the resistant strains, and the lysed culture as antigen.

A technic was developed for the quantitative estimation of bacteriophage concentration, using an original type isolated by d'Herelle.

The normal rate of multiplication of *Eberthella dysenteriae* (Shiga) during the logarithmic phase of growth was determined, the rate of fission being once every 31.3 minutes in beef infusion broth at 37 C.

The effect of the bacteriophage on this normal logarithmic rate is such as to cause no variation from the normal for a greater or lesser

period of time, depending on the concentrations both of the bacteriophage and of the organisms; following this there is a period of rapid lysis.

During this exposure of organisms to bacteriophage, a period of stability in bacteriophage concentration occurs, followed by a period of rapid increase, to some extent synchronous with the period of rapid bacterial lysis.

There was found to be a maximum concentration of bacteriophage in beef infusion broth, beyond which further lysis of bacterial cells seemed to cause no increase. This point of concentration was approximately 225,000,000 units per c c.

Experimental results on cellular absorption of the bacteriophage showed the following:

1. Living bacterial cells sensitive to the lytic action of the bacteriophage absorb bacteriophage from a broth suspension within a period of 15 minutes at 37 C. Dead cells of the same strain bring about progressive adsorption over a period of at least one hour. It was shown that such dead cells probably adsorb bacteriophage rather than absorb it.

2. Tests involving the absorption of bacteriophage by heterologous bacteria indicated absorption from a broth suspension within a period of 15 minutes at 37 C. with some bacterial strains and none with others.

3. It is concluded that there is no specificity of bacteriophage absorption from the point of view of the accepted bacteriologic grouping, but that there is a specificity from the point of view of susceptibility to lysis by the bacteriophage.

The bacteriophage was found to diffuse 1 mm. in 2% agar in 48 hours, and to diffuse through parchmented and collodion membranes. It is indicated that adsorption plays a rôle in the diffusion through parchmented membranes.

Evidence is given to indicate a probability that our resistant or R strains are combinations of normal cells, and older cells with a natural (possibly acquired) resistance, all associated with adsorbed and absorbed bacteriophage.

Attempts at adaptation of an antityphoid (Eberth) bacteriophage to a Rawling's strain of the typhoid bacillus were unsuccessful.

The use of typhoid culture subjected to lysis by bacteriophage as antigen in animal inoculation experiments did not result in a specific

antityphoid serum when tested for the presence of agglutinins and precipitins against the normal antigen and the lysed antigen.

Normal antityphoid immune serum, although having a high agglutinin concentration specific for our Eberth strain of the typhoid bacillus, was not found to have precipitins giving specific precipitation with bacteriophage-lysed *Eberthella typhi* (Eberth) antigen.

A discussion of the unity of structure of the bacteriophage is presented, and the theory that the agent responsible for transmissible bacteriolysis is a living ultramicroscopic entity is concurred in.

PLATE 1

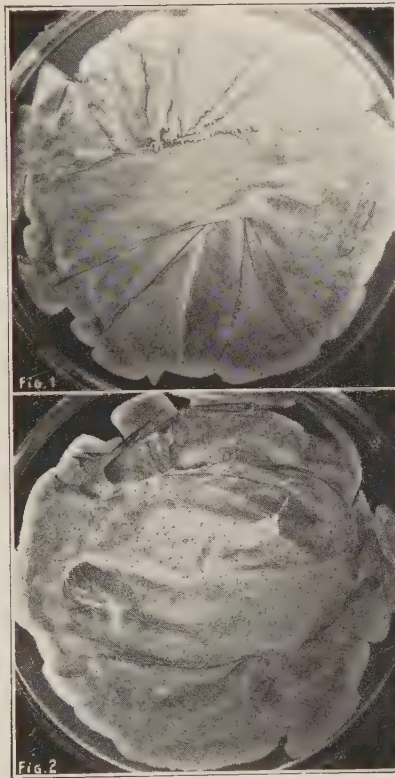


Fig. 1.—A standard plate used in enumeration of areas for determination of bacteriophage concentration. ($\times 0.6$)

Fig. 2.—Same as figure 1, with a dilution 0.1 as great. ($\times 0.6$)

Plate II

PLATE 2

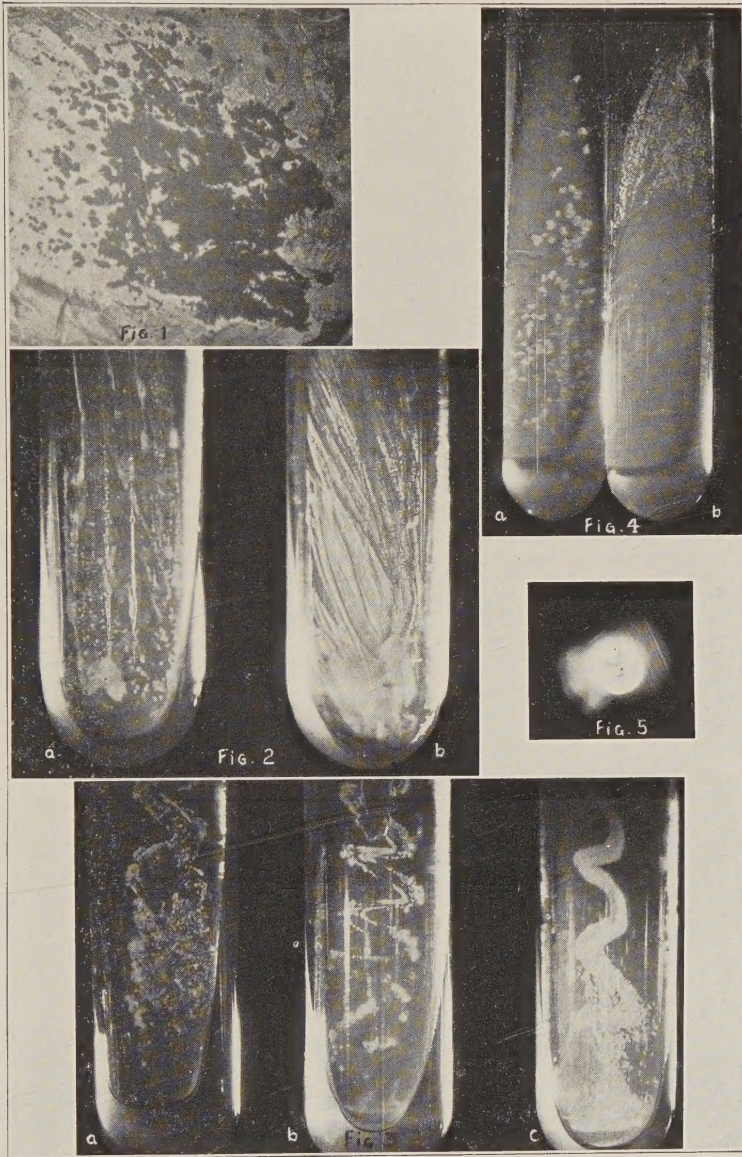


Fig. 1.—Washed areas resulting from excessive moisture on a plate used for enumeration work. (Natural size.)

Fig. 2.—(a) Agar slant culture of an R strain; (b) an agar slant on which a very young R strain culture was streaked over with normal culture, illustrating the presence of a large amount of bacteriophage on an R strain culture. ($\times 1.4$.)

Fig. 3.—(a) and (b) Irregular growth of R strain; (c) normal strain. ($\times 1.2$.)

Fig. 4.—(a) Production of "pie shaped" colonies by method described; (b) a similar agar slant heavily inoculated. ($\times 0.8$.)

Fig. 5.—Typical "pie-shaped" colony. ($\times 2$.)

